PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 06 March 2001 (06.03.01)	
International application No. PCT/DK00/00281	**Applicant's or agent's file reference** P375PC00
International filing date (day/month/year) 25 May 2000 (25.05.00)	**Priority date** (day/month/year) 25 May 1999 (25.05.99)
Applicant PHILIP, John et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

15 December 2000 (15.12.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer C. Cupello
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

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From the INTERNATIONAL BUREAU

To:

HØIBERG APS
Nørre Farimagsgade 37
DK-1364 Copenhagen K
DANEMARK

NOTIFICATION CONCERNING SUBMISSION OR TRANSMITTAL OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

Date of mailing (day/month/year) 14 September 2000 (14.09.00)	
Applicant's or agent's file reference P375PC00	**IMPORTANT NOTIFICATION**
International application No. PCT/DK00/00281	International filing date (day/month/year) 25 May 2000 (25.05.00)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 25 May 1999 (25.05.99)
Applicant PHILIP, John et al	

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

2. This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.

3. An **asterisk(*)** appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

4. The **letters "NR"** appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
25 May 1999 (25.05.99)	99201651.9	EP	18 Augu 2000 (18.08.00)

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer

Marc Salzman

Telephone No. (41-22) 338.83.38

TENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
P375PC00		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/DK00/00281	25/05/2000	25/05/1999
International Patent Classification (IPC) or national classification and IPC G01N33/50		
Applicant PHILIP, John et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 4 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
15/12/2000	06.09.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Bigot-Maucher, C Telephone No. +49 89 2399 7415

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/DK00/00281

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-15	as originally filed	

Claims, No.:

1-28	with telefax of	16/07/2001

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   - ☐ the description, pages:
   - ☐ the claims, Nos.:
   - ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-28
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-28
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-28
	No:	Claims	

2. Citations and explanations
see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

The arguments of the applicant have been taken into consideration for the establishment of this International Preliminary Examination Report.

Item V:

Reference is made to the following documents:

D1: EP-A-0 791 659
D3: WO-A-98 40746
D8: US-A-5 731 156, cited in the application

1. Articles 33(2) and 33(3) PCT

1.1. The subject-matter of **independent claim 1 appears to be novel** (Article 33(2) PCT), since none of the available prior art documents discloses a method for isolating fetal cells comprising the use of "a sample ... having a size of 0.5-40ml" and "from which at the most 50% of maternal nucleated and/or anucleated cells have been removed" (see however VIII, 1).

Due to the use of the expression "at the most 50%", present claim 1 includes the option wherein neither maternal nucleated, nor maternal anucleated cells are removed.

In D1 (p 4, l 38-39) or D8 (col 6, l 30) an isolation method for fetal cells is described using an enriched or a whole blood sample, i.e. a sample without prior removal of maternal nucleated and / or anucleated cells.

However, neither D1, nor D8 is discloses how a maternal whole blood sample, which by definition comprises only very few fetal cells, can be used for the isolation method. Said documents are therefore not considered enabling for the use of non-enriched samples.

The expression "at the most 50%" in present claim 1 also includes enriched

samples.

It appears that D1 and D8 only disclose methods wherein more than 50% of maternal cells are removed, since the following methods are used: Ficoll gradient density centrifugation method (removes all nucleated cells) and the use of 2 different antibodies (distinguish between fetal and maternal cells) (p 7, last par).

Thus, the technical problem to be solved in the light of the closest prior art D1 or D8 appears to be an improved method for isolating fetal cells wherein fewer fetal cells are lost during the procedure.

The problem is solved using a sample ... having a size of 0.5-40ml (i.e. a large sample) from which at the most 50% of maternal nucleated and/or anucleated cells have been removed, wherein the selectively labelled cells are identified by scanning with a scanning rate of from 0.1m/sec to 10m/sec (see VIII, 1).

Said solution to use a large sample with at the most removal of cells, and to use a fast scanner, which is necessary for large samples, could not be derived from any prior art document, either taken alone or in any combination. Therefore, **independent claim 1 appears to be inventive** (see however VIII, 1).

The same applies to dependent claims 2-24.

1.2. The subject-matter of **independent claim 25 is considered novel** (Article 33(2) PCT) in the light of the available prior art, since none of the documents discloses a method of diagnosing a disease in a fetus comprising the use of "a sample ... having a size of 0.5-40ml" and "from which at the most 50% of maternal nucleated and/or anucleated cells have been removed".

The following documents disclose methods of diagnosing a disease in a fetus using a disease marker by examining an enriched sample of blood (for the whole blood sample examination in D8 see 1.2, 3rd par herein above): D3 (p 5, l 5-9 and claim 12), D8 (col 2, l 32-50).

The technical problem to be solved in view of the closest prior art D3 or D8 is the provision of an improved method of diagnosing a disease in a fetus using a disease marker wherein less fetal cells are lost during the procedure.

The problem is solved using a sample having a size of 0.5-40ml from which at the most 50% of maternal nucleated and/or anucleated cells have been removed, wherein the selectively labelled cells are identified by scanning with a scanning rate of from 0.1m/sec to 10m/sec.

Said solution could not be derived from any prior art document, either if taken alone or in any combination (see also 1.1.). Therefore, the subject-matter of **independent claim 25 appears to be inventive**.

The same applies to dependent claims 26-28.

Item VII:

Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the documents D1, D3 and D8 is not mentioned in the description, nor are these documents identified therein.

Item VIII:

1. It is clear from the description on page 9, l 26-28 ("an important factor for the detection equipment is the rate of cells identified per unit of time") that the following feature is essential to the definition of the invention:

   "scanning with a scanning rate of from 0.1m/sec to 10m/sec"

   Since independent claims 1 and 25 do not contain this feature, they do not meet the requirement following from Article 6 PCT taken in combination with Rule 6.3(b) PCT that any independent claim must contain all the technical features essential to the definition of the invention.

PCT/DK00/00281
Philip, John et al.
P 375 PC00

Claims per July 16, 2001:

1. A method for isolating fetal cells from maternal blood, comprising

performing, on a sample of maternal blood having a size of from 0.5 to 40 ml, from which at the most 50 % of the maternal nucleated cells thereof have been removed, and/or at the most 50 % of the maternal anucleated cells have been removed,

selective labelling of fetal cells in the maternal blood sample,
identifying the selectively labelled fetal cells by scanning from 0.5 to 40 ml of the maternal blood sample, and

specifically isolating substantially only the selectively labelled fetal cells.

2. The method according to claim 1, wherein the selectively labelled cells are identified by scanning with a scanning rate of from 0.1 m/sec to 10 m/sec.

3. The method according to claim 1, wherein at the most 15 % of the maternal cells thereof have been removed.

4. The method according to claim 1, wherein at the most 10 % of the maternal cells thereof have been removed.

5. The method according to claim 1, wherein at the most 5 % of the maternal cells thereof have been removed.

6. The method according to claim 1, wherein at the most 2.5 % of the maternal cells thereof have been removed.

7. The method according to claim 1, wherein at the most 1% of the maternal cells thereof have been removed.

8. The method according to claim 1, wherein substantially none of the maternal cells have been removed from the sample.

9. The method according to claim 1, wherein at the most 20 % of the maternal nucleated blood cells thereof have been removed.

10. The method according to claim 1, wherein at the most 20 % of the anucleated red blood cells thereof have been removed.

11. The method according to the claim 1, wherein substantially none of the anucleated blood cells have been removed from the sample.

12. The method according to claim 1, wherein at the most 20% of the anucleated red blood cells have been removed from the sample, and at the most 20% of the nucleated blood cells have been removed from the sample.

13. The method according to any of the preceding claims, wherein the maternal blood sample is diluted before labelling or identification of the fetal cells.

14. The method according to any of the preceding claims, wherein the selective labelling is based on hybridisation of a probe to m-RNA selectively expressed by fetal cells.

15. The method according to claim 14, wherein the m-RNA is m-RNA coding for a protein selected from the group consisting of embryonic hemoglobin, such as epsilon globin chains and zeta globin chains, and fetal hemoglobin, such as gamma globin chains, and alpha globin chains.

16. The method according to claim 14 or 15, wherein the hybridisation probe is directly labelled by having fluorochromes covalently attached thereto .

17. The method according to any of claims 1-13, wherein the selective labelling is based on an antigen-antibody reaction with a protein selectively produced by fetal cells.

18. The method according to claim 17, wherein the protein is a protein selected from the group consisting of embroynic hemoglobin, such as epsilon globin chains and

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zeta globin chains, and fetal hemoglobin, such as gamma globin chains and alpha globin chains.

19. The method according to claim 17 or 18, wherein the antibody is selected from anti epsilon (ε) antibodies, such as monoclonal unlabelled antibodies, and monoclonal fluorochrome labeled antibodies, anti zeta (ζ) antibodies, such as monoclonal unlabelled antibodies, monoclonal biotin labelled antibodies, monoclonal fluorochrome labeled antibodies, and fluorochrome labeled antibodies, anti gamma (γ) antibodies, such as polyclonal (sheep) antibodies, such as monoclonal unlabelled antibodies, monoclonal biotin labelled antibodies, monoclonal fluorochrome labeled antibodies, and fluorochrome labeled antibodies, anti alpha (α) antibodies, and anti beta (β) antibodies.

20. The method according to any of the preceding claims, wherein two or more selective labellings are performed to enhance the probability of identifying the fetal cells in the sample.

21. The method according to claim 20, wherein a labelling with a hybridisation probe is combined with a antigen-antibody labelling.

22. The method according to any of the preceding claims, wherein the identification of the selectively labelled fetal cells is taking place after spreading the blood sample on a solid surface and detecting labelled cells on the surface.

23. The method according to claim 22 wherein the position of detected labelled cells on the surface is recorded.

24. The method according to claim 23, wherein the detected cells the position of which has been recorded are collected.

25. A method of diagnosing a disease in a fetus comprising

obtaining a blood sample having a size of from 0.5 to 40 ml from the woman pregnant with said fetus, whereby at most 50 % of the maternal nucleated cells have

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been removed and/or at most 50 % of the maternal anucleated cells have been removed from said blood sample,

selective labelling the fetal cells in the maternal blood sample,

identifying the selectively labelled fetal cells, by scanning from 0.5 to 40 ml of the maternal blood sample,

specifically labelling with at least one disease marker the identified fetal cells for diseases, and identifying specifically labelled cells.

26. The method according to claim 25, wherein the fetal cells are isolated before specifically labelling the fetal cells with at least one disease marker.

27. The method according to claim 25, wherein the disease is a genetic disease and/or a chromosome abnormality.

28. The method according to claim 27, wherein the disease and/or chromosome abnormality is cystic fibrosis, hemophilia, muscular dystrophy, Down' syndrome, Klinefelter, Turner' syndrome.

The demand must be filed directly with the competent International Preliminary Examining Authority or, if two or more Authorities are competent, with the one chosen by the applicant. The full name or two-letter code of that Authority may be indicated by the applicant on the line below:

IPEA/ EPO √

PCT

CHAPTER II

DEMAND

under Article 31 of the Patent Cooperation Treaty:
The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty and hereby elects all eligible States (except where otherwise indicated).

For International Preliminary Examining Authority use only

Identification of IPEA	Date of receipt of DEMAND

Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION		Applicant's or agent's file reference: P 375 PC00
International application No.: PCT/DK00/00281 √	International filing date *(day/month/year)*: 25. May 2000 (25.05.2000) √	(Earliest) Priority date *(day/month/year)*: 25. May 1999 (25.05.1999) √

Title of invention: Isolation and culturing of fetal cells

Box No. II APPLICANT(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*	Telephone No.:
Philip, John Berlingsbakke 11 DK-2920 Charlottenlund √	Facsimile No.:
	Teleprinter No.:

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
Denmark	Denmark

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

Christensen, Britta
Hovedgaden 38A, 2.tv.,
DK-3460 Birkerød √

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
Denmark	Denmark

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

State *(that is, country)* of nationality:	State *(that is, country)* of residence:

☐ Further applicants are indicated on a continuation sheet.

International application No.
PCT/DK00/00281

Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The following person is [x] agent [ ] common representative

and [x] has been appointed earlier and represents the applicant(s) also for international preliminary examination.

[ ] is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked.

[ ] is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

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Facsimile No.: +45 33 32 03 84

Teleprinter No.:

[ ] **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special addr ess to which correspondence should be sent.

Box No. IV BASIS FOR INTERNATIONAL PRELIMINARY EXAMINATION

Statement concerning amendments:*

1. The applicant wishes the international preliminary examination **to start on the basis of:**

[x] the international application as originally filed

the description [x] as originally filed
[ ] as amended under Article 34

the claims [x] as originally filed
[ ] as amended under Article 19 (together with any accompanying statement)
[ ] as amended under Article 34

the drawings [x] as originally filed
[ ] as amended under Article 34

2. [ ] The applicant wishes any amendment to the claims under Article 19 to be considered as reversed.

3. [ ] The applicant wishes the start of the international preliminary examination **to be postponed** until the expiration of 20 months from the priority date unless the International Preliminary Examining Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)). *(This check-box may be marked only where the time limit under Article 19 has not yet expired.)*

* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Language for the purposes of international preliminary examination: English

[x] which is the language in which the international application was filed.
[ ] which is the language of a translation furnished for the purposes of international search.
[ ] which is the language of publication of the international application.
[ ] which is the language of the translation (to be) furnished for the purposes of international preliminary examination.

Box No. V ELECTION OF STATES

The applicant hereby **elects all eligible States** *(that is, all States which have been designated and which are bound by Chapter II of the PCT)*

excluding the following States which the applicant wishes **not to elect:**

Sheet No. 3

International application No. PCT/DK00/00281

Box No. VI CHECK LIST

The demand is accompanied by the following elements, in the language referred to in Box No. IV, for the purposes of international preliminary examination:			For International Preliminary Examining Authority use only: received	For International Preliminary Examining Authority use only: not received
1. translation of international application	:	sheets	☐	☐
2. amendments under Article 34	:	sheets	☐	☐
3. copy (or, where required, translation) of amendments under Article 19	:	sheets	☐	☐
4. copy (or, where required, translation) of statement under Article 19	:	sheets	☐	☐
5. letter	: 1	sheets	☐	☐
6. other *(specify)*	:	sheets	☐	☐

The demand is also accompanied by the item(s) marked below:

1. [x] fee calculation sheet ✓
2. [ ] separate signed power of attorney
3. [ ] copy of general power of attorney; reference number, if any:
4. [ ] statement explaining lack of signature
5. [ ] nucleotide and or amino acid sequence listing in computer readable form
6. [ ] other *(specify)*:

Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).

Copenhagen 14 december 2000 Susanne Høiberg

For International Preliminary Examining Authority use only

1. Date of actual receipt of DEMAND:
2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):
3. ☐ The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply. ☐ The applicant has been informed accordingly.
4. ☐ The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5.
5. ☐ Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82.

For International Bureau use only

Demand received from IPEA on:

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
30 November 2000 (30.11.2000)

PCT

(10) International Publication Number
WO 00/71987 A2

(51) International Patent Classification[7]: G01N

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(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
99201651.9 25 May 1999 (25.05.1999) EP

(71) Applicants and
(72) Inventors: **PHILIP, John** [DK/DK]; Berlingsbakke 11, DK-2920 Charlottenlund (DK). **CHRISTENSEN, Britta** [DK/DK]; Hovedgaden 38A, 2.tv., DK-3460 Birkerød (DK).

(74) Agent: **HØIBERG APS**; Nørre Farimagsgade 37, DK-1364 Copenhagen K (DK).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *Without international search report and to be republished upon receipt of that report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ISOLATION AND CULTURING OF FETAL CELLS

(57) Abstract: The present invention relates to a method for isolating fetal cells from maternal blood, wherein the blood sample has not been substantially enriched, such as by performing, on a sample of maternal blood from which at the most 50 % of the anucleated material cells thereof and/or at the most 50 % of the nucleated maternal cells thereof have been removed, selective labelling of fetal cells in the maternal blood sample, identifying the selectively labelled fetal cells, and specifically isolating substantially only the selectively labelled fetal cells. The selective labelling may be conducted with antibodies specific for the fetal cells and/or probes hybridising for example with fetal mRNA. Also, the invention relates to a method of diagnosing a disease in a fetus comprising obtaining a blood sample from the woman pregnant with said fetus, whereby at most 50 % of the maternal nucleated cells have been removed and/or at most 50 % of the maternal anucleated cells have been removed from said blood sample, selective labelling the fetal cells in the maternal blood sample, identifying the selectively labelled fetal cells, specifically labelling with at least one disease marker the identified fetal cells for diseases, and identifying specifically labelled cells. The specifically labelling may then be conducted with a marker, such as a probe, to a gene or a gene mutation specific for the genetic disease or a chromosome abnormality to be diagnosed.

WO 00/71987 A2

Title

Isolation and culturing of fetal cells.

Introduction

The present invention relates to a method for isolating fetal cells from maternal blood, in particular from maternal blood not having been subjected to concentration or enrichment.

In addition the invention relates to a method for culturing fetal cells.

Background

The examination of fetal cells for early detection of fetal diseases and genetic abnormalities is carried out in connection with many pregnancies, in particular when the maternal age is high (35 years or above) or where genetic diseases are known in the family. Fetal cells may be obtained by amniocentesis, the removal of amniotic fluid from the amniotic cavity within the amniotic sac or by chorion biopsy, where biopsies are taken from the placenta, a so-called invasive sampling.

During pregnancy a variety of cell types of fetal origin cross the placenta and circulate within maternal peripheral blood. The feasability of using fetal cells in the maternal circulation for diagnostic purposes has been hindered by the fact that fetal cells are present in maternal blood in only very limited numbers, reported numbers have been from $1:10^5$ to $1:10^8$ fetal cells per nucleated maternal cells. In addition most fetal cells cannot be distinguished from maternal cells on the basis of morphology alone, but rather must be identified based upon detection of fetal cell markers. However, it would be advantageously to perform fetal diagnosis by a non-invasive procedure, such as a maternal blood sample.

One particular fetal cell type within maternal blood that has been demonstrated to be useful for detecting fetal DNA is the nucleated erythrocyte.

Also, fetal leukocytes have been reported to be present in maternal blood. Leukocytes are one subpopulation of white blood cells found in the blood. There are three types of subsets of leukocytes (which also are referred to as polymorphnuclear leukocytes): neutrophils, basophils and eosinophils. All leukocytes have a distinctiv morphology characterized by the nucleus and cellular granules.

Due to the very limited number of fetal cells in maternal blood concentration or enrichment of the maternal blood sample with respect to the fetal cells have been conducted by negative selection, i.e. removal of maternal cells. Enrichment of fetal cells by density gradient centrifugation or by removing maternal cells with an antibody to a cell surface antigen is described in for example US 5,858,649, US 5,731,156, US 5,766,843 and US 5,861,253.

Yet another method of removing maternal cells, in particular maternal erythrocytes, is by lysing, again optionally combined with immunologic methods for removing the maternal cells.

Another selection procedure is positive selection, for example by use of CD71 antibodies.

US 5,861,253 describes enrichment either before and/or after labelling of the fetal cells for further analysis.

It is however, a problem that due to the enrichment procedures some of the fetal cells may also be removed leading to even fewer fetal cells in the blood sample to be analysed.

In order to increase the number of fetal cells attempts of culturing the cells have been carried out in the prior art. There are a few publications describing successful methods for culturing fetal cells from peripheral blood of pregnant women. Lo et al, (Lancet 1994, 344, 264) cultured cells from five pregnant women carrying male fetuses. In two cases they examined samples before culturing with negative results. In all cases they ascertained cells by Fluorescence in situ hybridization (FISH) after culture. Very few details of the culture methodology are giv n, and the results have not been repeatable with the available information. In a study by Little et al, (Blood,

273, R 1829) culture was used as part of their isolation method and male cells were found after various sorting/enrichment procedures. Also Jansen t al, (Prenatal Diagnosis, 1999, 19, 323) developed a method for culturing cord blood cells in a model system for isolation of fetal nucleated red blood cells. However, none of the methods have shown a significant increase in fetal cells after culture.

Summary of the invention

It is an object of the present invention to provide for a method for isolating fetal cells from maternal blood, wherein the blood sample has not been substantially enriched, such as by performing, on a sample of maternal blood from which at the most 50% of the anucleated maternal cells thereof and/or at the most 50% of the nucleated maternal cells thereof have been removed, selective labelling of fetal cells in the maternal blood sample, identifying the selectively labelled fetal cells, and specifically isolating substantially only the selectively labelled fetal cells.

Thus, according to the invention a method is obtained whereby the risk of removing fetal cells from the maternal blood sample before analysing the sample has been greatly reduced.

Detailed description of the invention

The present invention reveals a novel method of isolating fetal cells from maternal blood. The present method has proven to be optimised in relation to isolating methods described in the prior art, and the present invention represents a method having beneficial properties technically and financially. In the light of the naturally occurring ratio between maternal cells and fetal cells the present invention presents a method wherein fetal cells are isolated from maternal blood without prior enrichment or concentration of the sample, providing for a method of isolation by which the risk of losing fetal cells due to enrichment or concentration procedure has been reduced.

A major difference between maternal and fetal red blood cells is the latter having a nucleus, i.e. maternal red blood cells are anucleated. Furthermore, maternal blood contains three types of nucleated fetal cells, nucleated erythrocytes, syncytiotrophoblasts and leukocytes. It is an object of the present invention to provide for a method

wherein the blood sample from which the fetal cells are isolated is substantially not enrich d or concentrated prior to isolation by removing any of the maternal cells.

Accordingly, in order to reduce the risk of removing fetal cells in the preparation of maternal blood samples to be analysed it is an object of the present invention that at most 50 % of the maternal cells of the maternal blood sample have been removed or will be removed before or after the labelling of the fetal cells, thus that substantially no enrichment of the sample is carried out before identification of the cells. In a more preferred embodiment at most 20 % of the maternal cells have been removed, such as at the most 15% of the maternal cells, such as at most 10 % of the maternal cells, more preferred at most 5 % of the maternal cells, more preferably at most 2.5 % of the maternal cells, most preferred at most 1 % of the maternal cells.

According to the invention it is even more preferred that substantially none of the maternal cells have been removed from the sample.

Thus, it is encompassed by the present invention that at most 20 % of the maternal nucleated blood cells have been or will be removed and/or at most 20 % of the anucleated red blood cells have been or will be removed.

Even more preferred is a method wherein substantially none of the nucleated blood cells or anucleated red blood cells have been removed from the sample. Thereby the sample may be used as such directly after taking the maternal blood sample.

It is desirable to obtain as large a maternal blood sample as possible in order to increase the total number of fetal cells. However, due to proctical problems the sample must be within certain limits. Accordingly, the size of the maternal blood sample is preferably in the range of 0,5 to 40 ml, such as in the range of 5 to 40 ml, such as from 10 to 30 ml.

Also, according to the invention it is preferred to dilute the sample before labelling or before identification of the fetal cells (to facilitate the identification of the fetal cells). The sample may be diluted at least 1.5 times, such as twice, more preferred at least three times, such as five times by adding isotonic buffers, such as saline solutions,

phosphate buffered saline solutions, PBS, and/or suitabl growth media, such as basal media, and tissues growth m dia.

The selective labelling of the fetal cells may be carried out by any suitable method. Fetal cells may be distinguished from maternal cells by the specific recognition of a fetal cell antigen or they may be distinguished by staining with a labelled antibody to a protein selectively produced by fetal cells or they may be distinguished from maternal cells by the specific recognition of DNA or RNA encoding a protein selectively or substantially selectively produced by fetal cells.

Accordingly, it is an object of the present invention to provide for the selective labelling of fetal blood cells in the maternal blood sample based on hybridisation of a probe to m-RNA selectively expressed by fetal cells.

Preferably, fetal-cell-specific RNA sequences are used as fetal cell markers. Such RNA is generally messenger RNA (mRNA). The presence of such RNA indicates that the gene for the fetal protein is being transcribed and expressed. (The probes used to identify fetal cells in a sample containing fetal and maternal cells include nucleic acid molecules, which comprise the nucleotide sequence complementary to the nucleotide sequence of the RNA molecule encoding a specific protein. Fetal cells contain distinct mRNAs or RNA species that do not occur in other cell types. The detection of these RNAs, whether as mRNA can serve to identify cells, or even subcellular fractions of cells fetal or embryonic in origin). According to the present invention the m-RNA to be detected may be coding for a protein selected from the group consisting of embryonic hemoglobin, such as ε and zeta globin chains, and fetal hemoglobin, such as gamma and alpha globin chains.

Further, according to the present invention DNA probes (oligos) for the hybridisation are directed against embryonic cell RNA, such as for ε and zeta globin chains, and for fetal hemoglobin, such as for gamma and alpha globin chains. A DNA probe may be synthesised as an oligodeoxynucleotide using a commercial synthesiser. Probes may be comprised of the natural nucleotide bases or known analogues of the natural nucleotide bases.

Yet further to the invention the hybridisation probe for the DNA or RNA discussed above is s lected from peptide nucl ic acid (PNA) probes and other synthetic molecules capable of Watson Crick-base pairing with the fetal m-RNA.

In one embodiment of the invention the probe as discussed above, such as a synthetic DNA probe, is directly labelled, by having fluorochromes covalently attached thereto. The binding of such probes to the cell may be observed under a microscope as a bright fluorescence or may be detected by a fluorimetric apparatus.

Instead of direct labelling or in addition to the direct labelling in another embodiment the probes are indirectly labelled with biotin or enzymes for example, such as alkaline phosphatase.

By using a combination of labelling methods it is possible to enhance the signals from the fetal cells, thereby facilitating the identification thereof.

Certain RNA populations are present in high abundance and other fetal or embryonic-specific RNAs are present in low abundance. Several RNA species occur simultanously in fetal cells as opposed to maternal cells. This provides for yet another method of enhancing the distinction between fetal cells and non-fetal cells by the detection of multiple RNA species. Two or more RNA species may be detected using one or more probes for a first RNA sequence and one or more probes for a second RNA sequence. The probes for the first sequence are labelled to provide a first signal, such as a greenish fluorescence, and the probes for the second sequence are labelled to provide a signal that is different from the first signal, such as a reddish fluorescence. When the combination of both signals are detected in a single cell, which in this case would be an orange fluorescence, then both RNAs are found and thus a fetal cell has been detected.

According to another method of the invention the selective labelling is based on an antigen-antibody reaction with a protein selectively produced by fetal cells. Such a protein may be selected from the group consisting of embryonic hemoglobin, such as ε and zeta globin chains, and fetal hemoglobin, such as gamma and alpha globin chains.

In particular the labelling may be carried out by the use of an antibody selected from antibodies against various types of normal globin chains in human hemoglobin, for example anti epsilon (ε) antibodies, such as monoclonal unlabelled antibodies, monoclonal biotin labelled antibodies, monoclonal biotin and fluorochrome labelled antibodies, and monoclonal fluorochrome labelled antibodies, anti zeta (ζ) antibodies, such as monoclonal unlabelled antibodies, monoclonal biotin labelled antibodies, monoclonal fluorochrome labeled antibodies, and polyclonal fluorochrome labeled antibodies, anti gamma (γ) antibodies, such as polyclonal (sheep) antibodies, such as monoclonal unlabelled antibodies, monoclonal biotin labelled antibodies, monoclonal fluorochrome labeled antibodies, and polyclonal fluorochrome labeled antibodies, anti alpha (α) antibodies, and anti beta (β) antibodies.

The fluorochrome is selected to be excited in the wave-length area of the detection means, and furthermore in suitable combination with an optional second labelling. In particular the fluorochromes may be selected from FITC (fluorescein-isofluocyanate) or TRITC (Rhodanine Tetramethyl- isofluocyanate) having excitation at 495 nm and 555 nm, respectively.

In a preferred embodiment of the present invention the labelling is carried out using anti epsilon (ε) monoclonal antibodies or anti zeta (ζ) monoclonal antibodies, more preferably anti epsilon (ε) unlabelled monoclonal antibodies or anti zeta (ζ) unlabelled monoclonal antibodies, or anti epsilon (ε) monoclonal biotin labelled antibodies or anti zeta (ζ) monoclonal biotin labelled antibodies.

The unlabelled antibodies are used as known in the art, by using a second labelling step with eg. second antibodies against the unlabelled antibody, said antibody being labelled as discussed above, such as fluorochrome labelled. By this two-step it may be possible to enhance the signals from the fetal cells.

In another preferred embodiment of the invention the labelling is performed using anti gamma (γ) fluorochrome labeled antibodies, such as FITC labelled antibodies.

In order to enhance the probability and selectivity of identifying the fetal cells or the background of maternal cells by the labelling two or more selective labellings may be performed. The combination of two or more labellings may be a combination of

any of the labellings used for single labelling as well. Accordingly, the combined labelling may b carried out by the use of two or more different hybridisation probes, such as a combination of a DNA probe and a PNA probe for hybridisation with the same fetal RNA or more preferred with different RNAs. Also, two or more different DNA probes (or PNA probes) may be used for hybridisation with different fetal RNAs.

The enhanced selective labelling may also by carried out by the use of two or more antibodies directed against the same protein or different proteins. In this embodiment the labelling with two or more labels may be carried out simultaneously.

In another embodiment a combination of an immunological labelling and a hybridisation labelling may be employed according to the present invention. In this embodiment the labelling is normally carried out sequentially by a first immunological labelling step, then identification of the labelled cells, and then a second step of hybridisation labelling for verification of the identification of the cells labelled by the first step. It is preferred in the first step to use antibodies against gamma globin and in the second step to use hybridisation for epsilon and/or zeta globin and/or gamma mRNA to verify the fetal cells identified.

In yet another embodiment the verification may be conducted by a second step of staining the cell nucleus since the maternal cells probable to be detected will be anucleated maternal cells, i.e. maternal erythrocytes. Thus by verifying that identified cells contain a nucleus it is ensured that fetal cells have been identified. In particular the nucleus staining is selected from 4,6-diamidino-2-phenylindole (DAPI) or propidium-iodide (PI).

An important feature of the present invention is the identification of the labelled fetal cells without the sample being enriched or concentrated with respect to the fetal cells in order to avoid loss of fetal cells. Accordingly, the method according to the present invention comprises identification of the selectively labelled fetal cells.

In one embodiment the identification is performed by spreading the blood sample on a solid surface and detecting the labelled cells on the surface. The detection may be carried out by any suitable means in accordance with the labelling method in ques-

tion. The choice of solid support surface may depend upon the procedure for visualisation of the cells. Some materials are not uniform and therefore shrinking and swelling during in situ hybridisation procedures will not be uniform leading to inaccuracy in the identification procedure. Other autofluoresce support materials will interfere with the determination of low level fluorescence. Support materials according to the invention preferably comprise glass, nylon, nitrocellulose and Scotch tape, and any suitable membranes, such as filtermembranes. Preferably, the collected samples are spread on a support surface in a monolayer for the cells not to overlap one another. Also, the in situ hybridization process according to the invention may carried out on fetal cells attached to a solid support.

Various antibodies have been used to discriminate between maternal and fetal cells as discussed above. In one embodiment the antibodies may be coupled to numerous solid surfaces or supports/substrates, such as containers, columns, wells, beads, or particles by physical or chemical bonding. Alternatively, the antibodies may be coupled to a compound which facilitates the separation step. For example antibodies may be labelled with fluorescent markers and cells to which these labelled antibodies bind may thereby be separated with a cell sorter according to known procedures.

The nucleic acid of the fetal cells can be amplified prior to detection using a known amplification technique, such as the polymerase chain reaction (PCR). Primers for PCR amplification are chosen to specifically amplify a DNA of interest in the fetal DNA.

Due to the large amount of cells to be examined to find the small amount of fetal cells in the blood sample an important factor for the detection equipment is the rate of cells identified per unit of time. For example very fast scanning microscopes may be used for the identification. Also, laser scanners could be used. Preferably the laser scanner is equipped with at least two lasers emitting light with different wavelength capable to excite the various labels on the cells or in one laser emitting several wavelengths.

Pref rably, during or after identification of the fetal cells the position of detected labelled cells on the surface is record d. This provides for th later collection of the detected cells from the position which has been recorded. The position of the detected labelled cells on the supporting surface may be recorded by use of a scanner provided with detectors registering the light emitted from the labelled cells, such photo-multipliers, CCDs, or the like detectors. Thereby it is possible to identify and specifically isolate substantially only the selectively labelled fetal cells.

In a preferred embodiment the scanner is arranged for detecting selectively labelled fetal cells, and when detecting a fetal cell, carrying out a verification step by switching to another wavelength to verify presence of for example staining of the nucleus. It is of importance for the use of the method that a fast scanning system is used, for example a scanner capable of scanning in the range of from 0.1 m/sec to 10 m/sec or faster, such as appr. 1m/sec.

The cells collected according to any of the procedures may be subjected to further identification and/or investigation, such as microscopic and/or molecular identification and/or investigation. The cells may be subjected to investigations of analysing the presence of genetic diseases, for example. The nucleic acid of fetal cells may be analysed for diagnostic or other purposes. For instance the presence or absence of a gene or a gene mutation may indicate the presence of diseases, such as cystic fibrosis. The nucleic acid may additionally be analysed for X or Y specificity. Thus, the presence of a Y chromosome encoded genes or gene products is a qualitative distinguishing feature of the cells of a male fetus.

Verification of the selective identification of fetal cells may be carried out by several methods. In a model system the method may be performed on maternal blood samples from pregnant women carrying a male fetus. The cells isolated may then be analysed for the presence of a Y chromosome, indicative of cells being from the male fetus.

Another verification method, which is usable independent of the sex of the fetus, is verification by use of identification of small tandem repeats (STR) or variable number tandem repeats (VNTR) to detect genetic input from the father, thereby verifying fetal cells, as the only cells in the sample comprising input from the father.

As may be understood from the above the present method may be carried out for the isolation of any kind of rare event cells in a blood sample, and is particular interesting when used for rare event cells being present in very low concentrations, such as those for the fetal cells in maternal blood. This may for example also be true for some cancer forms.

Further, according to the present invention a method for multiplying fetal blood cells, preferably fetal red blood cells, in a cell culture comprising fetal blood cells and other cells, in particular maternal blood cells may be employed. The cultivation may be carried out on any sample comprising fetal blood cells, such as umbilical cord samples from aborted fetuses, or maternal blood samples.

The multiplication of the fetal cells is desirable to achieve due to the use of the present invention. The method according to the invention comprises performing the cell culturing in a culture medium containing growth factors necessary for the multiplication of fetal cells.

The multiplication may be conducted by any suitable method as known to the person skilled in the art.

The method of culturing fetal cells may be combined with the method of selectively isolating fetal cells in any suitable manner. Accordingly, a maternal blood sample may be subjected to cultivation of fetal cells before subjecting the sample to labelling, identification and isolation of the fetal cells. Thereby the starting concentrations of fetal cells in the maternal blood sample are increased without removing any cells originating from the mother. The culture method may also be used in connection with analysis of maternal blood samples that have been pretreated in order to remove some of the maternal cells.

When the labelling and identification procedures allow viable cells to be selected the culture method may be conducted on fetal cells having been identified and isolated according to the isolation method described above, in order to increase the amount of fetal cells for further analysis.

After cultivation the fetal cells may be harvested by any suitable method. In case the colonies obtained are harvested collectively, there will be a very high number of cells of which only some will be from the fetus. Contrary to this, colonies may be harvested individually to increase the rate of fetal cells in the harvested material.

Also, the invention relates to a method of diagnosing a disease in a fetus comprising obtaining a blood sample from the woman pregnant with said fetus, whereby at most 50 % of the maternal nucleated cells have been removed and/or at most 50 % of the maternal anucleated cells have been removed from said blood sample, selectively labelling the fetal cells in the maternal blood sample, identifying the selectively labelled fetal cells, specifically labelling with at least one disease marker the identified fetal cells for diseases, and identifying specifically labelled cells.

The selective labelling and identification of the cells are carried out as described above. The specific labelling with at least one disease marker refers to the labelling of the identified cells with a marker, such as a probe, to a gene or a gene mutation specific for the genetic disease or a chromosome abnormality to be diagnosed.

In a preferred embodiment wherein the fetal cells are isolated before specifically labelling the fetal cells.

Furthermore, the present invention relates to the use of the present method for diagnosing a disease of the fetus, such as a genetic disease. The genetic disease may be any genetic disease or a chromosome abnormality, such as cystic fibrosis, hemophilia, muscular dystrophy, Down' syndrome, Klinefelter, or Turner' syndrome.

The invention is further exemplified by the following non-limiting examples.

Examples

Example 1

Fixation of peripheral venous blood cells before mounting on slides.

The blood is fixed according to table I below and mounted on slides as described.

Tabl I

St p	Proc dure: Fixati n and slide pr parati n.	Time	T mp ra-ture
1	100 µl blood. Ad 100 µl Hank's (Ca^{++} and Mg^{++} free) or PBS		R.T.
2	Ad 200 µl 4% paraformaldehyde in PBS (Vortex)	1 hr	37 °C
3	Ad 3 ml PBS (+,- 3%BSA)		R.T.
4	Spin at 1500 rpm	5 min.	R.T.
5	Permeate cells with 1 ml methanol/acetone (1+1, -20°C)(Vortex)	1 hr - o.n*	4 °C
6	Ad 3 ml cold PBS		4°C
7	Spin at 1500 rpm	5 min	R.T.
8	Wash cells in cold PBS (+,- 3%BSA).		4°C.
9	Spin at 1500 rpm	5 min	R.T.
10	Resuspend cells in PBS (aprox. 100 µl) (+,- 3% BSA)		R.T.
11	Prepare slides. Smears on poly-L-lysine coated slides.		R.T.
12	Store slides sealed individually in plasticbags		-80 °C

* o.n. = over night.

The slides prepared are stained with antibodies against globin chains and embryonic globin chains as described below in table II.

Table II

Step	Procedure: Antibody staining of slides	Time	Tempera-ture
1	Wash slides in PBS	2 x 5 min	R.T.
2	Wask slides in 4xSSC	10 min	R.T.
3	Block in 4xSSC/1%BSA/0,5% Boehringer blocking reagent = Buffer A	10 min	R.T.
4	Inkubate with primary (anti-globin chain antibody, 2-3 µg/slide) diluted in buffer A.	30 min humid atm.	R.T.
5	Wash in 4xSSC/0,5% Tween20	2 x 5 min	R.T.
6	Inkubate with biotinylated goat anti-mouse diluted in buffer A.	30 min humid atm.	R.T.
7	Wash in 4xSSC/0,5% Tween 20	2 x 5 min	R.T.
8	Inkubate with fluorochrome conjugated avidin/streptavidin diluted in buffer A	30 min humid atm.	R.T.
9	Wash in 4xSSC/0,5%Tween 20	2 x 5 min	R.T.
10	Wash in 2xSSC	5 min	R.T.
11	Mount in Vectashield with counterstain added (DAPI or PI)		R.T.
12	Store slides in darkness		4 °C

Vectashield is a trademark to V ctor Laboratories, USA.

After staining the slides may be stored for later identification of the fetal cells. The identification may be carried out in a laser scanner.

Example 2

Mounting on slides before fixation.

Pheripheral venous blood is obtained from the pregnant woman.

Slides are prepared by smearing the blood on poly-L-lysine coated slides, whereafter the slides are stored individually in plastic bags at –20 °C. The fixation is carried out as described in table III.

Table III

Step	Procedure: Fixation.	Time	Temperature
1	Fix cells in 2% paraformaldehyde in PBS.	10 min	RT
2	Permeabilize cells methanol/acetone (1:1)	10 min	-20 °C

Antibody staining and identification is as described in example 1. The cell morphology is maintained more consistently when mounting on slides before fixation is carried out.

Example 3

Verification of fetal cells by identifying the Y chromosomes

Fetal cells were diagnosed by the Y chromosome FISH analysis by the following procedure:

Maternal whole blood is smeared onto a slide, and fixed 2 minutes in paraformaldehyde (2 %) at room temperature. The cells are permeabilised 10 minutes in acetone at –20 °C, and then washed 3 minutes in PBS-buffer at room temperature. The slides are dehydrated in 62%, 96% and 99% alcohol at room temperature, and air-dried. A DNA probe 'pBAM-X' for the X-chromosome labelled with digoxigenin and a DNA probe 'ph-y 2,1' for the Y-chromosome labelled with biotin are used. The slide is placed on a heating plate at 37 °C, and the probe is added to the slide and a

cov r glass placed on top of it and sealed with glue. The slide is placed immediately on a h ating plate at 83,5 °C in 7 minutes, and hybridisation is conducted over night at 42 °C in moistened air.

After hybridisation the glue is removed and the slide is washed in 2xSSC until the cover glass releases, the in 0.4xSSC/0.3% NP-40 in 2 minutes at 73 °C. Then in 2xSSC/0.1% NP-40 in 1 minute at room temperature, and then rinsed in 2xSSC.

The slide is incubated 20 minutes at 37 °C with fluorescein-labelled avidin/rhodamin labelled anti-digoxigenin, and then washed 5 minutes in 4xSSC/0.1% tween 20, rinsed in 2xSSC and air dryed.

The slide is then mounted in Vectashield with counterstain added (DAPI or PI).

The identification of cells is as described in example 1.

The verification method may alternatively be a diagnostic method using probes relevant for the diseases and/or chromosome abnormalities to diagnose.

Claims:

1. A method for isolating fetal cells from maternal blood, comprising performing, on a sample of maternal blood from which at the most 50 % of the maternal nucleated cells thereof have been removed, and/or at the most 50 % of the maternal anucleated cells have been removed, selective labelling of fetal cells in the maternal blood sample, identifying the selectively labelled fetal cells, and specifically isolating substantially only the selectively labelled fetal cells.

2. The method according to claim 1, wherein at the most 15 % of the maternal cells thereof have been removed.

3. The method according to claim 1, wherein at the most 10 % of the maternal cells thereof have been removed.

4. The method according to claim 1, wherein at the most 5 % of the maternal cells thereof have been removed.

5. The method according to claim 1, wherein at the most 2.5 % of the maternal cells thereof have been removed.

6. The method according to claim 1, wherein at the most 1% of the maternal cells thereof have been removed.

7. The method according to claim 1, wherein substantially none of the maternal cells have been removed from the sample.

8. The method according to claim 1, wherein at the most 20 % of the maternal nucleated blood cells thereof have been removed.

9. The method according to claim 1, wherein at the most 20 % of the anucleated red blood cells thereof have been removed.

10. The method according to the claim 1, wherein substantially none of th anucleated blood cells have been removed from the sample.

11. The method according to claim 1, wh rein at the most 20% of the anucleated red blood cells have been removed from the sample, and at the most 20% of the nucleated blood cells have been removed from the sample.

12. The method according to any of the preceding claims, wherein the maternal blood sample is diluted before labelling or identification of the fetal cells.

13. The method according to any of the preceding claims, wherein the selective labelling is based on hybridisation of a probe to m-RNA selectively expressed by fetal cells.

14. The method according to claim 13, wherein the m-RNA is m-RNA coding for a protein selected from the group consisting of embryonic hemoglobin, such as and zeta globin chains, and fetal hemoglobin, such as gamma and alpha globin chains.

15. The method according to claim 13 or 14, wherein the hybridisation probe is selected from DNA probes, PNA probes, and other synthetic molecules capable of Watson Crick-base pairing with the fetal m-RNA.

16. The method according to any of claims 1-12, wherein the selective labelling is based on an antigen-antibody reaction with a protein selectively produced by fetal cells.

17. The method according to claim 18, wherein the protein is a protein selected from the group consisting of embroynic hemoglobin, such as and zeta globin chains, and fetal hemoglobin, such as gamma and alpha globin chains.

18. The method according to claim 16 or 17, wherein the antibody is selected from anti epsilon (ε) antibodies, such as monoclonal unlabelled antibodies, and monoclonal fluorochrome labeled antibodies, anti zeta (ζ) antibodies, such as monoclonal unlabelled antibodies, monoclonal biotin labelled antibodies, monoclonal fluorochrome labeled antibodies, and fluorochrome labeled antibodies, anti gamma (γ) antibodies, such as polyclonal (sh ep) antibodies, such as monoclonal unlabelled antibodies, monoclonal biotin labelled antibodies, monoclonal fluorochrom labeled

antibodies, and fluorochrome labeled antibodies, anti alpha (α) antibodi s, and anti beta (β) antibodies.

19. The method according to any of the preceding claims, wherein two or more selective labellings are performed to enhance the probability of identifying the fetal cells in the sample.

20. The method according to claim 19, wherein a labelling with a hybridisation probe is combined with a antigen-antibody labelling.

21. The method according to any of the preceding claims, wherein the identification of the selectively labelled fetal cells is taken place after spreading the blood sample on a solid surface and detecting labelled cells on the surface.

22. The method according to claim 21 wherein the position of detected labelled cells on the surface is recorded.

23. The method according to claim 22, wherein the detected cells the position of which has been recorded are collected.

24. A method of diagnosing a disease in a fetus comprising obtaining a blood sample from the woman pregnant with said fetus, whereby at most 50 % of the maternal nucleated cells have been removed and/or at most 50 % of the maternal anucleated cells have been removed from said blood sample, selective labelling the fetal cells in the maternal blood sample, identifying the selectively labelled fetal cells, specifically labelling with at least one disease marker the identified fetal cells for diseases, and identifying specifically labelled cells.

25. The method according to claim 24, wherein the fetal cells are isolated before specifically labelling the fetal cells with at least one disease marker.

26. The method according to claim 24, wherein the disease is a genetic disease and/or a chromosome abnormality.

27. The method according to claim 26, wherein the disease and/or chromosome abnormality is cystic fibrosis, hemophilia, muscular dystrophy, Down' syndrome, Klinefelter, Turner' syndrome.

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WO 00/71987 A3

(54) Title: ISOLATION AND CULTURING OF FETAL CELLS

(57) Abstract: The present invention relates to a method for isolating fetal cells from maternal blood, wherein the blood sample has not been substantially enriched, such as by performing, on a sample of maternal blood from which at the most 50 % of the anucleated material cells thereof and/or at the most 50 % of the nucleated maternal cells thereof have been removed, selective labelling of fetal cells in the maternal blood sample, identifying the selectively labelled fetal cells, and specifically isolating substantially only the selectively labelled fetal cells. The selective labelling may be conducted with antibodies specific for the fetal cells and/or probes hybridising for example with fetal mRNA. Also, the invention relates to a method of diagnosing a disease in a fetus wherein the specifically labelling may then be conducted with a marker, such as a probe, to a gene or a gene mutation specific for the genetic disease or a chromosome abnormality to be diagnosed.

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT 00/00281

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/50 G01N33/569 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 791 659 A (CHILDRENS MEDICAL CENTER) 27 August 1997 (1997-08-27) claims page 4, line 25 - line 43 page 5, line 11 - line 12	1-27
P,X	WO 99 41613 A (RAO GALLA CHANDRA ;TERSTAPPEN LEON W M M (US); IMMUNIVEST (US); LI) 19 August 1999 (1999-08-19) claims 1-6,48-50 page 16, line 16 - line 30 page 17, line 28 -page 18, line 26 page 28, line 12 - line 24	1-27

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 December 2000	21/12/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Routledge, B

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International Application No PCT/[redacted] 00/00281

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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X	WO 95 09245 A (ONCOR INC) 6 April 1995 (1995-04-06) claims 1,7,8 page 6, line 18 - line 28 page 14, line 7 - line 17 example 2	1-27
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X	US 5 447 842 A (SIMONS MALCOLM J) 5 September 1995 (1995-09-05) claims column 3, line 3 - line 30 column 3, line 49 -column 4, line 6 column 6, line 8 - line 39	1-27

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